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Our studies, along with studies from other laboratories, have indicated that defects in the cyclin D1-Rb-p16 cell cycle regulatory system are present in the vast majority of breast cancer cells. In the case of cyclin D1, defects in expression are associated with increased amplification of the cyclin D1 gene and increased levels of mRNA expression, as well as, in one case, a change in the half-life of the protein. Overexpression of cyclin D1 has also been shown to be independent of growth factor regulation and largely unaffected by cell-cell contact. Failure to express p16 has been shown to be due to homologous deletion or methylation of the p16 gene in 70 percent of breast cancer cell lines. In order to evaluate the contribution of failure to express p16 to the tumorigenicity of breast cancer cell lines, we have now expressed p16 under the control of the inducible Tet promoter, and plan to test the consequences of p16 expression on the ability of these cells to form and maintain tumors in nude mice. These studies on the fundamental defects in cell cycle regulatory mechanisms in breast cancer cells, on the mechanisms underlying these defects, and on approaches to correcting such defects will provide for an increased understanding of oncogenesis in breast cancer and may provide targets for future therapy.

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Thos A. Longon 10/18/97

PI - Signature Date

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Introduction:

In the first three years of the grant, as described previously and in the present annual report, we have established a panel of 12 available breast cancer and normal breast epithelial cell lines, and characterized them with respect to the expression of a total of 13 cell cycle regulatory proteins by immunoblotting. We also examined 10 matched pairs of normal breast and breast tumor tissue for the expression of Rb (retinoblastoma) cyclin D1 and p16 (multiple tumor suppressor 1) proteins. These studies, along with studies from other laboratories, have indicated that one or more defects in the Rb-cyclin D1-p16 cell cycle regulatory system are present in a large majority of breast cancer cells. We have also investigated the mechanisms that lead to overexpression of cyclin D1 and the failure to express p16 in breast cancer. Cyclin D1 expression is associated with amplification of the cyclin D1 gene and increases in cyclin D1 mRNA expression as well as, in one case, an increase in the half-life of the cyclin D1 protein. In addition, expression of cyclin D1 protein in breast cancer cell lines is independent of growth factor regulation, and largely unaffected by cell-cell contact. Failure to express p16 has been shown to be due to either homozygous deletion or methylation of the gene in 70 percent of breast cancer cell lines. We have now expressed p16 protein under the control of the inducible Tet promoter in breast cancer cells lacking p16 production, and will test the effect of restoration of p16 expression on the tumorigenicity of these cells. We have also initiated a study of cyclin D1, CDK4 and CDK6-associated kinase activity in breast cancer cells.

Knowledge of the mechanisms of cell cycle regulation and the techniques available to investigate it have undergone very large advances since our original proposal was written. We have accordingly altered and expanded our original goals, in order to bring our investigation into currency with the present state of knowledge, as reflected in the revised Statement of Work submitted earlier this year. In particular, technology for the transfection and expression of genes in cells has advanced considerably, and we have recently chosen to exploit one of these advances for the introduction of genes affecting cell cycle regulation into breast cancer cells. However, these changes involve changes only in the reagents that we plan to use to accomplish one of the principal goals of the original proposal, and reflect the availability of new and potentially more advantageous ways to achieve those goals.

Materials and Methods

Breast cancer cell lines and tumor material

Three breast cancer cell lines, MCF-7, MDA-MB-231, and ZR75.1, and one normal, immortalized, non-transformed breast epithelial cell line, MCF-12A were obtained from the University of Colorado Tissue Culture Core Facility. MCF-12A was cultured in Ham's F12/DME (1:1) supplemented with 10% fetal calf serum, 20 ng/ml EGF, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin and 10 µg/ml insulin. The remaining cell lines were cultured in MEM media supplemented with 10% fetal calf serum, 0.2% sodium bicarbonate, 10 mM HEPES, 1% non-essential amino acids, 2 mM L-glutamine and 6 ng/ml insulin. An additional six pairs of frozen normal and tumor tissues from breast cancer patients were obtained by Dr. Wilbur Franklin (University of Colorado Cancer Center Tissue Procurement Core) bringing the total size of the breast tissue panel to ten pairs.

Antibodies

The following primary antibodies were obtained from Upstate Biotechnology, Lake Placid, NY: anti-cyclin D1, anti-CDK2, anti-CDK4, anti-CDK6, and anti-PSTAIRE kinase. Anti-p16 was obtained from Pharmingen. Anti-pRb was a gift from Dr. Wen-Hua Lee (University of Texas Health Science Center, San Antonio, TX) The horseradish peroxidase-conjugated secondary antibodies were obtained from Bio-Rad.

Oligonucleotides

The pUDH15-1 olignucleotide primer sequences are as follows:

Sense primer: 5' TAG ATG TGC TTT ACT AAG TC 3'
Antisense primer: 5' ACT TGA TGC TCT TGA TCT TC 3'

Protein extraction and western blot analysis

Cells were harvested, washed in PBS, and resuspended in Laemmli sample buffer (Laemmli., 1970). The extracts were then boiled for 4 minutes, sheared through a 26-gauge syringe needle, aliquoted, and stored at -80°C. Individual pieces of tumor and normal tissues each weighing approximately 0.2 g, were crushed to a fine powder under liquid nitrogen, lysed in Laemmli sample buffer and processed as described above for the cell lines.

Approximately 50-100 µg of each protein extract were subjected to SDS/PAGE and transferred either to nitrocellulose (Schleicher and Schuell) or Immobilon P (Amersham) membranes for 45 minutes at 0.45 A using the Genie Electrophoretic Blotter (Idea Scientific, Minneapolis). Membranes were stained with Ponceau dye to control for equal loading and immunodetection performed using the enhanced chemiluminescence (ECL) kit (Amersham) according to the manufacturer's instructions.

DNA isolation

DNA was prepared by incubating cells or finely minced solid tissues at 55°C in lysis buffer (10 mM Tris pH 8.0, 2.0 mM EDTA pH 8.0, 10 mM NaCl, 5% SDS) containing 1 mg/ml Proteinase K. The samples were then subjected to two phenol-chloroform extractions and one chloroform:isoamylalcohol (24:1) extraction, followed by ethanol precipitation.

DNA analysis by Methylation-specific PCR (MSP)

DNA samples were modified with sodium bisulfite according to the method of Herman et al. (1996), and precipitated with ammonium acetate (3M final concentration) and two volumes of ethanol. The resulting templates were subjected to PCR using oligonucleotides designed from the promoter of the p16 gene (Herman et al., 1996) specific for wildtype, methylated or unmethylated DNA. A 20 µl reaction mixture overlaid with a drop of mineral oil contained a final concentration of 50 ng of genomic DNA, 120 ng of each oligonucleotide, PCR buffer supplied by Promega (10 mM tris-HCL, pH 8.3; 5 mM KCl; 0.1% gelatin); 200 µM dNTPs; 1.5 mM $MgCl_2$; and 0.06 units μl^{-1} Taq polymerase (added once the reaction temperature reached 95°C). The DNA was subjected to 35 cycles of amplification consisting of denaturation for 0.5 minutes at 94°C, annealing for 0.5 minutes at 60°C (for unmethylated-specific oligonucleotides) or 65°C (for wildtype- and methylated-specific oligonucleotides), and elongation for 0.5 minutes at 72°C, followed by a final elongation step of 10 minutes at 72°C. The PCR products were subjected to electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized with uv light.

Purification of GST-Rb-(C) substrate

A 10 ml culture of E.coli, transformed with the plasmid expressing the GST-Rb-(C) fusion protein, was grown to saturation overnight. The cells were then used to seed a 500 ml culture which was grown for 2 hours at 30°C. Expression of the GST-Rb-(C) protein was induced by the addition of 1 mM IPTG and incubation at 30°C overnight. The cells were then harvested and lysed on ice by sonication in STE (50 mM Tris pH 7.5, 120 mM NaCl, and 1 mM EDTA) containing 1 mM DTT, 0.1 mM PMSF, 5 ug/ml leupeptin, and 1 mM pepstatin. Precleared lysates were then mixed with 50% glutathione-sepharose overnight at 4°C. The beads were washed once with 10 ml STE/1% triton containing 1 mM DTT and 100 mM PMSF, once with 10 ml STE containing 1 mM DTT and 100 mM PMSF, twice with 10 ml STE containing 0.5 mM DTT and 50 mM PMSF, and twice with 10 ml 50 mM HEPES containing 0.5 mM DTT and 50 mM PMSF. The GST-Rb-(C) protein was eluted in four stages using 0.5 ml kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA) containing 10 mM B-glycerophosphate, 0.1 mM sodium orthovanadate and 2 mM reduced glutathione. Ten microliter aliquots of each of the four elution products were subjected to electrophoresis on a 10% polyacylamide gel with protein standards of known concentration to assess both the substrate purity and concentration.

Immune complex kinase assays

Logarithmic phase cells were harvested from one 10 cm plate with 1 ml of HEPES/Tween lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM DTT, 0.1% Tween-20) containing 10 mM B-glycerophosphate, 0.1 mM sodium orthovanadate, 2 ug/ml aprotinin, 5 ug/ml leupeptin and 0.1 mM PMSF, and snap frozen in liquid nitrogen. Following thawing on ice, the lysates were clarified by centrifugation at 15 k rpm for 10 minutes at 4°C. Aliquots of the lysates were quantitated using the BCA protein assay kit (Pierce) and the remaining lysates precleared by mixing each milligram of protein with 20 ul of Protein A beads for 30 minutes at 4°C. Immunoprecipitation reactions were then performed by mixing 250 ug of each lysate with 20 ul of a 50/50 slurry of antibody precoated Protein A beads, and

incubation for 2 hours at 4°C. As a negative control, each lysate was also immunoprecipitated with Protein A beads precoated with normal rabbit serum. The immunoprecipitated protein on the beads was then washed four times with 1 ml of cold HEPES/Tween lysis buffer, and two times with 1 ml of cold 50 mM HEPES pH 7.5/1 mM DTT. The beads were then resuspended in 30 ul of kinase buffer (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA) containing, 10 mM B-glycerophosphate, 0.1 mM sodium orthovanadate, 20 uM cold ATP, 10 uCi gamma ^{32}P(ATP) and 1 ug GST-Rb-(C). The reactions were incubated at 30°C for 30 minutes with occasional mixing, boiled in 30 ul 2 x SDS-PAGE loading buffer, and fractionated on a 10% SDS-polyacrylamide gel. After Coomassie staining, the gel was dried down and exposed for autoradiography.

Transfection assays

Transfection assays were performed in triplicate on cells at 50-60% confluence in 6-well plates. For each well, 2.4 ug plasmid DNA were mixed with 12 ul of lipid in serum-free media, and incubated at room temperature for 15 minutes. The cells were then washed with 1 ml serum-free media and overlaid with the DNA/lipid mixture. After 5 hours at 37°C, the DNA/lipid mixture was removed and replaced with medium supplemented with 10% serum plus or minus 1 ug/ml doxycycline. The cells were then grown for a further 24 hours at 37°C and harvested for protein extraction and luciferase assays.

Luciferase assays

Cells were harvested with 1 ml PBS/1 mM EDTA/0.1% SDS per well. They were then lysed in 100 ul of 100 mM KH_2PO_4 pH 7.8 and 1 mM DTT, resuspended with vortexing, and subjected to three rounds of freezing in liquid nitrogen followed by thawing at 37°C. After each thaw, the cells were vortexed for 1 minute. Following centrifugation for 10 minutes at 15 k rpm at 4°C, the supernatant was transferred to a fresh tube and kept on ice. Two 30 ul aliquots of each extract were added to 400 ul of assay buffer (100 mM KH_2PO4 pH 7.8, 1 mM DTT, 15 mM $MgSO_4$ and 5 mM ATP) to which 100 ul of luciferin (1 mM luciferin, 100 mM KH_2PO_4 pH 7.8 and 1 mM DTT) was added automatically by the luminometer. A printout of relative light units was then obtained for each sample.

Results:

Primary breast tumor tissues exhibit a consistent correlation between Rb expression and cyclin D1 protein overexpression

The previous progress report presented the analysis of a panel of twelve breast cancer cell lines and four pairs of normal and tumor breast tissues for the expression of cell cycle regulatory proteins. Although the majority of proteins examined showed relatively constant expression levels, we observed consistent abnormalities in the expression of the G1-phase specific proteins, Rb, cyclin D1 and p16. Both the cell lines and the tumors demonstrated a clear correlation between cyclin D1 and Rb protein expression. Furthermore, six of the nine cell lines and all three of the tumors that expressed Rb protein, showed abnormally high levels of cyclin D1 protein relative to the normal breast cell line and tissue controls. With one exception, all of the cell lines that coexpressed Rb and cyclin D1 proteins failed to express p16 protein. These data suggested that the combined loss of p16 and moderate to overexpression of cyclin D1 protein is a mechanism frequently employed by breast cancer cells to overcome the Rb-mediated G1/S block.

To further investigate the pattern and frequency of aberrant G1 protein expression in primary breast tumors, we expanded the number of normal and tumor pairs from four to ten. The western analysis of the additional six pairs of tissues supported that observed in the original four pairs in that all tumors showed a positive correlation between Rb and cyclin D1 protein expression. Specifically, five of the six tumor tissue extracts expressed Rb protein and elevated expression of cyclin D1 protein relative to the corresponding normal tissue controls. The sixth tumor, COBRC8(T), expressed undetectable levels of both cyclin D1 and Rb proteins (Table 1). These data combined with that of the breast tumor cell lines reviewed above and those of other groups (Gillett et al., 1994; Zhang et al., 1994; Bartkova et al., 1994; Bartkova et al., 1995; Tam et al., 1994; Zukerberg et al., 1995) are consistent with an oncogenic basis for the overexpression of cyclin D1 in breast cancer.

Since p16 expression is not readily detectable by western analysis in breast tissue, we attempted to analyze its expression in the 10 pairs of normal and tumor tissue by immunohistochemistry. However, we have been unable to obtain reproducible staining patterns from the corresponding paraffin-embedded sections of the breast tumors and normal breast tissue controls. Owing to the technical difficulties associated with the analysis of p16 protein in primary breast tumors, we are planning to obtain RNA from microdissected samples of each of the tissues and analyze the expression of p16 by RT-PCR. Since all mechanisms of p16 inactivation reported to-date directly effect gene transcription (Kamb et al., 1994; Nobori et al., 1994; Herman et al., 1995; Herman et al., 1996; Gonzalez-Zulueta et al., 1995; Merlo et al., 1995), it is highly probable that the presence or absence of a p16 product by RT-PCR will directly correlate with the expression status of the p16 protein in the breast tissues.

Expression of cyclin D1 protein in breast cancer cell lines is serum-independent

The previous report showed that the mechanism of cyclin D1 overexpression in the breast cancer cell lines involved both the amplification and elevated transcription of the gene. In addition, one of the cell lines, ZR75.1, demonstrated post-translational stabilization of the cyclin D1 protein, an observation recently reported in two uterine sarcoma cell lines by Weleker et al. (1996). These observations strongly suggested the active oncogenic role of cyclin D1 overexpression, and the possibility that the expression of cyclin D1 was no longer regulated by environmental growth factors and serum in breast cancer cells. To address the latter possibility, we cultured all nine of the breast tumor cell lines that expressed moderate to elevated levels of cyclin D1 and the normal breast epithelial cell line, MCF-12A, in the absence of serum for one, two or three days respectively. Following protein extraction, the cell lines were analyzed for the expression of cyclin D1 by immunoblot analysis. Unlike normal macrophage cells that fail to sustain expression of cyclin D1 in the absence of serum (Hunter and Pines., 1994), none of the breast cancer cell lines nor the normal breast epithelial cell line MCF-12A, demonstrated a decrease in cyclin D1 protein expression in response to serum deprivation (Figure 1). The absence of a requirement for mitogenic stimulation of cyclin D1 expression may therefore represent a step toward cellular immortalization by removing a regulatory barrier to cyclin D1 expression and the subsequent uncontrolled progression through G1 into S-phase.

Expression of cyclin D1 protein in breast cancer cell lines is largely independent of cell contact-mediated negative regulation.

The apparent independence of cyclin D1 expression from external growth cues in the breast cancer cell lines was further investigated by assessing the effect of cellular confluence upon its expression. Three of the breast cancer cell lines that showed 10-fold overexpression of cyclin D1 (MCF-7, ZR75.1 and MDA-MB-330) and the normal epithelial cell line, MCF-12A, were cultured to different degrees of confluency: 50% confluency (cells in logarithmic phase of growth), 85% confluency, and 100% confluency. Extracts prepared from each set of culture conditions were analyzed for the expression of cyclin D1 protein by immunoblot analysis. The normal MCF-12A cell line showed a reduction in the expression of cyclin D1 to barely detectable levels once the cells reached 100% confluence, indicating that contact inhibition caused an essentially complete repression of cyclin D1 expression (Figure 2). In contrast, the breast cancer cell line ZR75.1 showed no reduction in cyclin expression upon reaching confluence (Figure 2), while MCF-7 and MDA-MB-330 cells exhibited 50% or less reduction (data not shown), such that cyclin D1 was still markedly overexpressed in these cells at confluence compared to normal MCF-12A cells growing logarithmically. The lack of substantial reduction in the level of cyclin D1 in the confluent breast cancer cell lines is most likely attributable to a loss of normal cell contact-mediated negative regulation of cyclin D1 expression in these cells.

Mechanisms of p16 inactivation in breast cancer

The p16 gene is methylated in 30 percent of breast cancer cell lines

Since ten of the twelve breast cancer cell lines did not express the p16 protein, we investigated the possible mechanisms by which it had been inactivated. The previous report showed by duplex PCR analysis that the p16 gene, that maps to 9p21, was homozygously deleted in 40 percent of the breast cancer cell lines. To determine the mechanism of p16 inactivation in the remaining 60 percent of cell lines that failed to express the p16 protein, we assessed them for methylation of the p16 gene. Cell line DNA was modified overnight using sodium bisulfite to convert all of the unmethylated cytosines to uracil. The resulting DNA was then subjected to PCR analysis using oligonucleotides designed from the promoter of the p16 gene that were specific for wild type (unmodified), methylated and unmethylated DNA (Herman et al., 1996). Three of the four cell lines that were previously shown to have undergone the homozygous deletion of the p16 gene (COLO 591, MCF-7, Hs578T) were included as negative controls for amplification. Two of the cell lines, DU4475 and T-47D demonstrated amplification using the methylated-specific oligonucleotides only, while a third cell line, ZR75.1 showed amplification using both methylated- and unmethylated-specific oligonucleotides (Figure 3). The latter result indicates either that the p16 promoter is hemi-methylated in ZR75.1 or that ZR75.1 is composed of a heterogeneous cellular population. The remaining three cell lines that lacked p16 expression but were not homozygously deleted for the p16 gene, underwent amplification with the oligonucleotides specific for unmethylated DNA only. Thus, 70 percent of the cell lines that did not express the p16 protein had undergone either the homozygous deletion or methylation of the gene. This is consistent with the findings of others in various types of human cancer (Kamb et al., 1994; Nobori et al., 1994; Liu et al., 1995; Herman et al., 1996; Cairns et al., 1994). The remaining 30 percent of cell lines lacking p16 protein are likely to have undergone mutation of the p16 gene and deletion of the remaining wildtype allele. In the absence of highly polymorphic markers within the p16 gene and the corresponding normal cell line counterpart to each of the remaining three cell lines, it is not possible to assess them for loss of heterozygosity at the p16 locus. Therefore, we plan to analyze the copy number of the p16 gene in the cell lines by Southern blot analysis using a probe to p16 followed by densitometric quantitation of the p16-specific hybridizing band. The signal in the three cell lines will be compared to that of MCF-12A, which has been shown to contain two normal copies of chromosome 9.

IP-Kinase Analysis of cyclin D1 complexes in breast cancer

Our immunoblot analysis of breast cancer cell lines and tissues has indicated that breast cancer cells overcome the Rb-mediated barrier to S-phase and uncontrolled proliferation through two major mechanisms: (1) inactivation of Rb through mutation in approximately 25 percent of cases; and (2) moderate to overexpression of cyclin D1 combined with the elimination of p16 in approximately 75 percent of cases. To determine whether breast cancer cells that exhibit the second

class of G1 protein expression defects listed above produce constitutively active cyclin D1/CDK4/CDK6 holoenzyme and therefore have the potential to inactivate Rb, we have initiated analysis of kinase activity in cyclin D1/CDK4/CDK6 complexes using a glutathione S-transferase-Rb-(C) fusion protein as substrate (gift from Dr. J. Gregori, Dept. of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center). The fusion protein consists of the C-terminus of Rb fused to GST resulting in a molecule of 45 kdal. Following purification of the GST-Rb-(C) protein (described in Materials and Methods), aliquots of each of the four sequential elution products were analyzed for concentration and purity by Coomassie blue staining of electrophorectically separated proteins on a SDS-polyacrylamide gel in comparison to carbonic anhydrase protein standards of known concentration (Figure 4).

We choose the breast cancer cell line, MCF-7, for the preliminary analysis of cyclin D1 complex kinase activity since it expressed Rb, showed 10-fold overexpression of cyclin D1 relative to the normal breast epithelial cell line, MCF-12A, and lacked p16 expression. The obligate CDK partners of cyclin D1, CDK4 and CDK6, were expressed at very low and moderate levels, respectively, in this cell line (as described in the first Progress report). Lysates of MCF-7 were prepared as described in the Materials and Methods and immunoprecipitated with antibodies to cyclin D1, CDK4 and CDK6 (all of which are components of cyclin D1 complexes in mid G1 phase), CDK2 (which is associated with cyclin E and cyclin A complexes in late G1 and early S-phase respectively), and normal rabbit serum (as a negative control). The immunoprecipitates were then assayed for kinase activity using GST-Rb-(C) as the substrate (Figure 5). Our preliminary analysis showed a high level of kinase activity in the CDK2 immunoprecipitate but a very low level of activity in the cyclin D1, CDK4 and CDK6 immunoprecipitates. Low levels of kinase activity in cyclin D1 and CDK4 immunoprecipitates from cyclin D1-overexpressing non-small cell lung cancer (NSCLC) cell lines have been observed by Grimison and Sclafani (unpublished results), suggesting that the currently available antibodies for cyclin D1 and CDK4, unlike those for CDK2, are not optimal for the detection of kinase activity despite their usefulness in immunoblot analysis. Although a low level of CDK4-associated kinase activity in MCF-7 cells is to be expected from the low level of CDK4 protein detected in these cells, there are substantial levels of CDK6 protein present, and it seems unlikely that rapidly growing cells would have low levels of all forms of cyclin D1-activated kinase activity. We are therefore investigating whether the low level of CDK6-associated kinase activity found is due to technical problems associated with preliminary optimization of IP-kinase assays in the breast cancer cell line, to the choice of antibodies, or perhaps to a combination of these factors. We are in the process of optimizing our assays using cell lysates from a NSCLC cell line, NCI H460, (with demonstrable cyclin D3, CDK4, CDK6 and CDK2 kinase activity), and the breast cancer cell lines, MCF-7 and ZR75.1 (both of which show Rb expression, 10-fold elevated expression of cyclin D1, and no p16 expression). The normal, immortalized breast epithelial cell line, MCF-12A, will also be studied to enable direct comparison with the transformed cell lines. The breast cancer cell line MDA-MB-231 (which lacks both Rb and cyclin D1 expression but expresses p16

protein) will be used as the negative control cell line for kinase activity. Experiments are also underway to identify the components of the cyclin D1 complexes in the above cell lines by analysis of cyclin D1 immunoprecipitates for the presence of Rb, CDK4, CDK6, p21 and PCNA by immunoblot analysis.

Regulated expression of sense p16 and antisense cyclin D1 in breast cancer cell lines

The patterns of expression of Rb, cyclin D1 and p16 in the breast cancer cell line panel strongly indicate the role of p16 inactivation and the subsequent unregulated phosphorylation of Rb by constitutively active cyclin D1/CDK4/CDK6 complexes, in the development of breast cancer. Indeed, suppression of cyclin D1 overexpression using an antisense cyclin D1 construct in a human esophageal cell line and a mouse lung cancer cell line was shown to result in inhibition of tumor formation in nude mice (Zhou et al., 1995; Schrump et al., 1996). In addition, the restoration of p16 expression in NSCLC cell lines lacking endogenous p16 activity has also been shown to reduce in vivo tumorigenicity (Jin et al., 1995). These experiments indicate that the correction of either the cyclin D1 or p16 expression defect is sufficient to suppress tumor formation from esophageal and lung cancer cells in nude mice. A major goal of this investigation, as proposed in our original grant application, has been to demonstrate similar suppression of tumor formation in nude mice by correction of defects in cyclin D1 or p16 expression in breast cancer cells. Our proposed approach was based on technology available at the time, and involved the use of expression vectors containing constitutive promoters to produce continuous high level expression of the vector encoded genes, either sense p16 or antisense cyclin D1 in the tumor cells. A potential disadvantage of this approach is that high levels of expression of these components might completely block cell growth in culture, prior to their injection into mice, thus preventing a test of their effects on the tumorigenicity of otherwise viable cells. However, since that time new technology superior to that originally proposed for expressing genes in cells has become available, and we chose instead to employ these newer procedures for expressing sense p16 or antisense cyclin D1 in breast cancer cells.

As described in the previous progress report, we began construction of a recombinant adenoviral vector, 327_{BST} B-gal, to express either sense p16 or antisense cyclin D1 under the control of a constitutive promoter. Unlike the retroviral or plasmid vectors used above, however, we anticipated that the overlap recombination between the adenoviral vector and the plasmid constructs, carrying either the p16 or antisense cyclin D1 genes (PACCMVpLpA-sense p16 and PACCMVpLpA-antisense cyclin D1) would result in random sequence rearrangements within the CMV promoter, such that the resulting recombinants would display a range of promoter strengths, some of which could be expected to be compatible with growth in culture. In addition, the very high efficiency of adenoviral vector-mediated transfection would allow transplantation of the cells into nude mice without the long period of growth in culture needed to select transfectants from a plasmid transfected population. Although we were successful in the construction of plasmids containing both the sense p16 and the antisense cyclin D1 genes respectively, we were unable, after

repeated attempts, to obtain recombinants between 327_{BST} B-gal and the PACCMVpLpA plasmid constructs. We initially decided, therefore to return to the use of the plasmid constructs originally proposed to transfect breast cancer cells. However, more recently the Tet expression system became readily available to us as a result of ongoing studies in our laboratories on the regulation of cyclin D1 and p16 in lung cancer cells.

The Tet expression system (Gossen et al., 1995) enables the highly regulated expression of genes in response to tetracycline or its derivative, doxycycline. We have chosen to use the Tet-Off system whereby gene expression (either in the sense or antisense orientation) is turned off in the presence of doxycycline to introduce either sense p16 or antisense cyclin D1 into the breast cancer cell lines, MCF-7, ZR75.1 and MDA-MB-231. Following transfection of the cell lines with constructs containing either p16 or antisense cyclin D1, the cells are cultured in the presence of doxycycline prior to subcutaneous injection into nude mice where the genes are maintained in the "off" state by the addition of doxycycline to the animal food source. Once tumors of 1 cm x 1 cm develop, the doxycycline supplements are removed from the food source thus allowing p16 and antisense cyclin D1 genes to be expressed. It is then possible to assess the roles of p16 expression and inhibition of cyclin D1 expression in the suppression of tumorigenesis. We believe that the use of the Tet system in the proposed experiments will more realistically mimic the clinical situation of a patient diagnosed with an established cancerous growth undergoing gene therapy.

Characteristics of the Tet expression system

The two plasmid vector components of the Tet system were kindly provided by Dr. Bujard, Heidelberg, Germany. The gene of interest is first cloned into a "response" plasmid, pTET-SPLICE, under the control of the Tet Operator (tet O) from the tet resistance operon of E. coli Tn 10, and the minimal immediate early promoter of CMV. In E. coli, the tet repressor protein (tet R) tightly binds the tet O except in the presence of tetracycline or doxycycline. A second "regulator" plasmid, pUDH15-1, expresses a hybrid protein called the tet-controlled transcriptional activator (tTA) which consists of the tet repressor DNA binding domain fused to the VP16 activation domain of herpes simplex virus. tTA binds the tet O sequence, and activates transcription of the target genes in the absence of tetracycline or doxycycline.

p16 and antisense cyclin D1 Tet constructs

A 0.5 kb p16 fragment was cloned into the HindIII-SpeI sites of the "response" plasmid, pTET-SPLICE in the sense orientation; and a 1.1 kb cyclin D1 fragment was cloned into the HindIII-SpeI sites of the "response" plasmid, pTET-SPLICE in the antisense orientation, by M. Dalton and R. A. Sclafani. The "regulator" plasmid, pUDH15-1, was modified by the cloning of the neomycin-resistance gene into the XhoI site to enable G418 selection.

Choice of breast cancer cell lines for transfection assays

The cell lines chosen for the introduction of antisense cyclin D1 and p16 were ZR75.1 and MCF-7 since both showed 10-fold overexpression of cyclin D1 and lack p16 expression. We presented the analysis of the tumorigenicity of these cell lines in the previous progress report and showed that of the two, only ZR75.1 was tumorigenic in nude mice. However, we have since learnt that MCF-7 cells will induce tumors in nude mice in the presence of estrogen. Since we were eager to perform these experiments on more than one cell line with the appropriate G1 protein expression profile, we decided to incorporate MCF-7 into our study. The cell line MDA-MB-231, will be used as a negative control in the transfection assays since it lacks cyclin D1 protein, expresses p16 protein, and is tumorigenic in nude mice.

Optimization of lipid-mediated transfection

We chose the highly efficient lipid-mediated transfection method to introduce the Tet system constructs into our breast cancer cell lines. Initially we tested a panel of 8 different lipids (Invitrogen) for their ability to transfect the pCMV-Luc plasmid (containing the luciferase gene under a constitutive promoter) into the MCF-7 and ZR75.1 cell lines. The relative transfection efficiencies mediated by each lipid were judged by assay of luciferase expression after incubation for 24 hours at 37°C (see Materials and Methods). Of the eight lipids, several showed 10^4 to 10^5-fold greater luciferase activity than the non-transfected control. The highest level of activity was demonstrated by cells transfected using lipid number 7. This particular lipid was therefore chosen for all further transfection experiments.

Transient transfection of MCF-7 and ZR75.1 with sense p16 and antisense cyclin D1 constructs

To ensure that the tet system vector constructs were functioning correctly in our breast cancer cell lines prior to producing stable transfectants, we initially performed transient transfections of MCF-7 and ZR75.1 with the sense p16 and antisense cyclin D1 constructs.

<u>p16 transient transfections</u>

The transfection assays were performed upon MCF-7 and ZR75.1 cells in 6-well plates, during logarithmic growth. The cells were transfected with (a) 2.4 ug/well of both the pTET-SPLICE-p16 (response plasmid) and the pUDH15-1 (regulatory plasmid); (b) 2.4 ug/well of the pTET-SPLICE-p16 plasmid, as a negative control; and (c) 2.4 ug/well of the pCMV-Luc plasmid, as a control for the lipid-mediated transfection efficiency. Cells were also left untransfected as a further negative control. After 5 hours at 37°C the DNA/lipid mixture was aspirated off the cells and replaced with medium containing 10% serum plus or minus 1ug/ml doxycycline. Following 24 hours at 37°C, the appropriate cells were harvested and assessed for p16 and luciferase expression. The lipid-mediated transfection efficiency was shown to be high

based upon analysis of luciferase expression in the pCMV-Luc transfected cells. This implied that the Tet system plasmids should also have been transfected at a high efficiency. Indeed, immunoblot analysis revealed that in those cells transfected with both pTET-SPLICE-p16 and pUDH15-1, the expression of p16 was "on" in the absence of doxycycline and "off" in the presence of doxycycline (Figure 6). These data confirm that the regulation of p16 expression by doxycycline worked successfully in each of the two breast cancer cell lines. Further those cells either transfected with pTET-SPLICE-p16 alone, or left untransfected, showed no expression of p16 in the presence or absence of doxycycline indicating the absolute requirement for tTA protein encoded by pUDH15-1 to allow p16 expression.

Antisense cyclin D1 transient transfections

The transient transfection of MCF-7 and ZR75.1 cell lines with the Tet system antisense cyclin D1 construct was performed as described above for the Tet system sense p16 construct. Whereas in the p16 experiment our aim was turn "on" the expression of the gene and thus express the protein, the goal of the antisense cyclin D1 experiment was to turn "on" the expression of the antisense gene, and thus suppress the expression of the cyclin D1 protein. Since the MCF-7 and ZR75.1 cell lines show 10-fold overexpression of cyclin D1 protein, we decided to transfect higher concentrations of each of the two DNA constructs than those used in the p16 transfections, in an attempt to express sufficient antisense cyclin D1 for the complete inhibition of cyclin D1 expression. Logarithmic cells were transfected with the following: (a) either 2.4, 24 or 36 ug/well of both the pTET-SPLICE-antisense cyclin D1 (response plasmid) and the pUDH15-1 (regulatory plasmid); (b) 2.4, and 24 ug/well of the pTET-SPLICE-antisense cyclin D1 plasmid, as a negative control; and (c) 2.4 ug/well of the pCMV-Luc plasmid, as a control for the lipid-mediated transfection efficiency. Cells were also left untransfected as a further negative control. After 5 hours at 37°C the DNA/lipid mixture was aspirated off the cells and replaced with medium containing 10% serum plus or minus 1ug/ml doxycycline. Following the growth of cells for 24 hours, they were harvested and assayed either for luciferase activity or p16 expression by immunoblot analysis. The degree of luciferase expression in those cells transfected with pCMV-Luc indicated that the transfection efficiency was as high as that observed in the previous p16 transient transfection experiment. However, we did not observe the expected suppression of cyclin D1 expression in those cells cotransfected with the three different amounts of the pTET-SPLICE-antisense cyclin D1 and pUDH15-1 plasmids, in the absence of doxycycline. It is likely that the elevated degree of mRNA transcription that lead to the 10-fold overexpression of cyclin D1 in both the MCF-7 and ZR75.1 cell lines was too great to be significantly reduced even by the increased concentrations of antisense cyclin D1 produced by the pTET-SPLICE-antisense cyclin D1 construct in the transfectants. We therefore, intend to optimize the transfections both by using even greater concentrations of the "regulator" and "response" plasmids, and by varying the lipid:DNA ratios to obtain even greater transfection efficiencies.

Stable transfection of MCF-7 and ZR75.1 with the "regulator" plasmid, pUDH15-1

The production of a "double stable" cell line wherein both the regulator plasmid (pUDH15-1) and the response plasmids (pTET-SPLICE-p16 or -antisense cyclin D1) are integrated in the genome, requires two independent transfection experiments, the first involving the stable integration of the regulator plasmid into each of the cell lines. As mentioned earlier, we cloned the neomycin-resistance gene into the pUDH15-1 plasmid to allow selection for those cells with resistance to G418. Prior to transfecting MCF-7 and ZR75.1 with pUDH15-1, we performed G418 "kill-curve" assays on the two cell lines with concentrations ranging from 300 to 1000 ug/ml. The minimum concentration required to kill all untransfected cells within one week was determined to be 500 ug/ml. Two 6-well plates containing MCF-7 and ZR75.1 cells were grown to 50-60% confluence at which time ten of the twelve wells were transfected with 2.4 ug/well of the pUDH15-1 plasmid encoding the tTA. Two wells were left untransfected. After 5 hours at 37°C, the lipid/DNA mixture was removed from the cells and replaced with medium containing 10% serum. The cells were grown for a further 48 hours at 37°C before each well (including the two wells containing untransfected cells) was harvested and its cells transferred to one 100 mm dish containing medium supplemented with 10% serum and 500 ug/ml G418. Every 5-7 days, the medium was changed and after two weeks each dish contained approximately 15-20 discrete colonies. As expected the two plates containing untransfected cells were killed in the presence of G418. We are in the process of cloning individual colonies and expanding them in culture. To confirm that the clones actually contain the pUDH15-1 plasmid, we will prepare DNA from each of them and subject it to PCR analysis using oligonucleotide primers to sequences within the tTA domain of the plasmid (see Materials and Methods). Once we have identified positive clones, we will undertake to stably transfect them with either the pTET-SPLICE-p16 or pTET-SPLICE-antisense cyclin D1, using hygromycin as the additional selection agent. At this time, doxycycline will also be added to the media to inhibit the expression of the sense p16 or antisense cyclin D1 genes until their expression is required for (a) *in vitro* soft agar assays to investigate anchorage independence; and (b) *in vivo* assays of tumorigenesis in nude mice.

Conclusions:

Our studies, along with studies from other laboratories, have indicated that defects of one type or another in the cyclin D1-Rb-p16 cell cycle regulatory system are present in the vast majority of breast cancer cells. In the case of cyclin D1, defects in expression are associated with increased amplification of the cyclin D1 gene and increased levels of mRNA expression, as well as, in one case, a change in the half-life of the protein. Our observation of increased protein stability associated with overexpression of cyclin D1, recently seen in one other type of cancer, represents the first observation of this mechanism in breast cancer cells. Overexpression of cyclin D1 has also been shown to be independent of growth factor regulation and largely unaffected by cell-cell contact. Failure to express p16 has been shown to be due to homologous deletion or methylation of the p16 gene in 70 percent of breast cancer cell lines. In order to evaluate the contribution of failure to express p16 to the tumorigenicity of breast cancer cell lines, we have now expressed p16 under the control of the inducible Tet promoter, and plan to test the consequences of p16 expression on the ability of these cells to form and maintain tumors in nude mice. These studies on the fundamental defects in cell cycle regulatory mechanisms in breast cancer cells, on the mechanisms underlying these defects, and on approaches to correcting such defects will provide for an increased understanding of oncogenesis in breast cancer and may provide targets for future therapy.

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Table 1 Expression of cyclin D1 and Rb proteins in primary breast cancer tissues

Tissue	Histology	Rb protein	Cyclin D1 protein
COBRC1 (N)	Normal epithelial	+	+
COBRC1 (T)	Ductal carcinoma	+	+++
COBRC2 (N)	Normal epithelial	+	+
COBRC2 (T)	In situ Ductal carcinoma	+	++++
COBRC3 (N)	Normal epithelial	+	+
COBRC3 (T)	Carcinoma	+	+++
COBRC4 (N)	Normal epithelial	+	-/+
COBRC4 (T)	Medulla carcinoma	-/+	-/+
COBRC5 (N)	Normal epithelial	+	+
COBRC5 (T)	unknown	+	+++
COBRC6 (N)	Normal epithelial	+	+
COBRC6 (T)	unknown	+	++
COBRC7 (N)	Normal epithelial	+	+
COBRC7 (T)	unknown	+	+/++
COBRC8 (N)	Normal epithelial	+/-	-
COBRC8 (T)	unknown	-	-
COBRC9 (N)	Normal epithelial	+	+
COBRC9 (T)	unknown	+	+/++
COBRC10 (N)	Normal epithelial	+	+
COBRC10 (T)	unknown	+	+++

The analysis of the six pairs of normal and tumor tissues, COBRC5 through COBRC10, for the expression of Rb and cyclin D1 proteins has been added to that of the four pairs of tissues, COBRC1 through COBRC4, presented in the previous progress report. The analysis of the six new tumor specimens was performed as a "blind" study to enable any possible correlations between histological type of tumor and G1 protein expression patterns to be assessed at a later date.

The presence or absence of Rb is indicated by a (+) a (-) respectively. The levels of cyclin D1 protein are indicated as follows: (++++), very high; (+++), high; (++), moderate; (-/+), barely detectable; (-), undetectable, relative to the corresponding normal breast tissue controls.

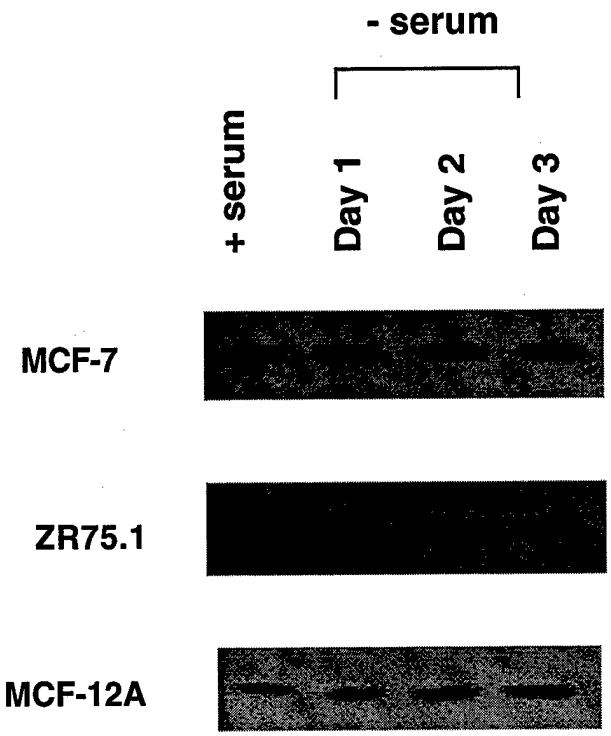

Figure 1 Effect of serum-deprivation upon cyclin D1 protein expression in pRb-positive breast cancer cell lines.
The normal breast epithelial cell line, MCF-12A and each of the nine breast cancer cell lines that express cyclin D1 were grown in the absence of serum for 1, 2 and 3 days respectively. Extracts were then prepared, subjected to SDS-PAGE, transferred to Immobilon P membranes and probed with an antibody to cyclin D1. Two representative breast cancer cell lines are shown, as is the normal breast epithelial cell line, MCF-12A.

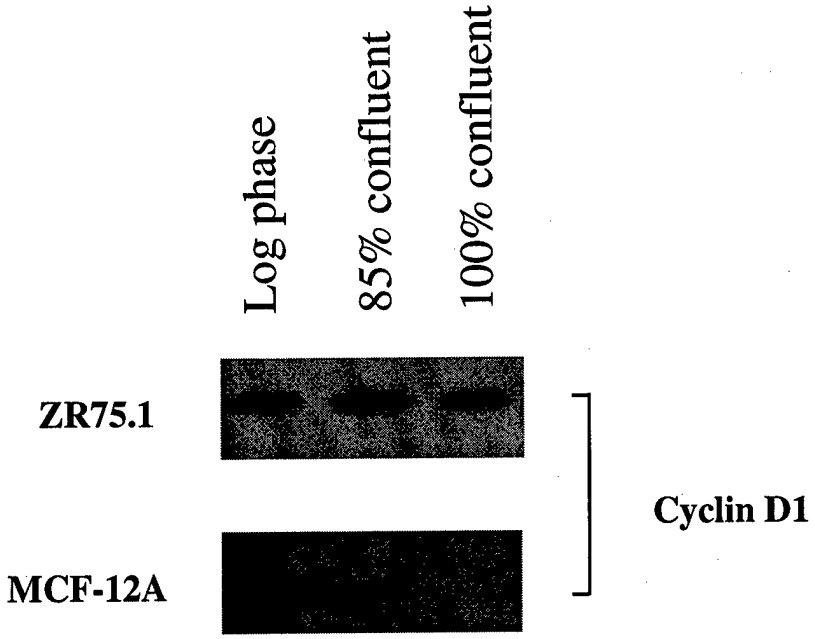

Figure 2 Effect of cellular confluence upon cyclin D1 expression. The normal breast epithelial cell line, MCF-12A, and three breast cancer cell lines (that showed 10-fold overexpression of cyclin D1), MCF-7, MDA-MB-330 and ZR75.1, were grown to 50% confluence (lane 1), 85% confluence (lane 2) and 100% confluence (lane 3). Extracts from each of the cultures were subjected to SDS-PAGE, transferred to Immobilon P, and probed with an antibody to cyclin D1. The figure contrasts the significant decrease in cyclin D1 expression in the 100% confluent MCF-12A culture compared to that of the confluent ZR75.1 culture where the elevated levels of cyclin D1 were maintained.

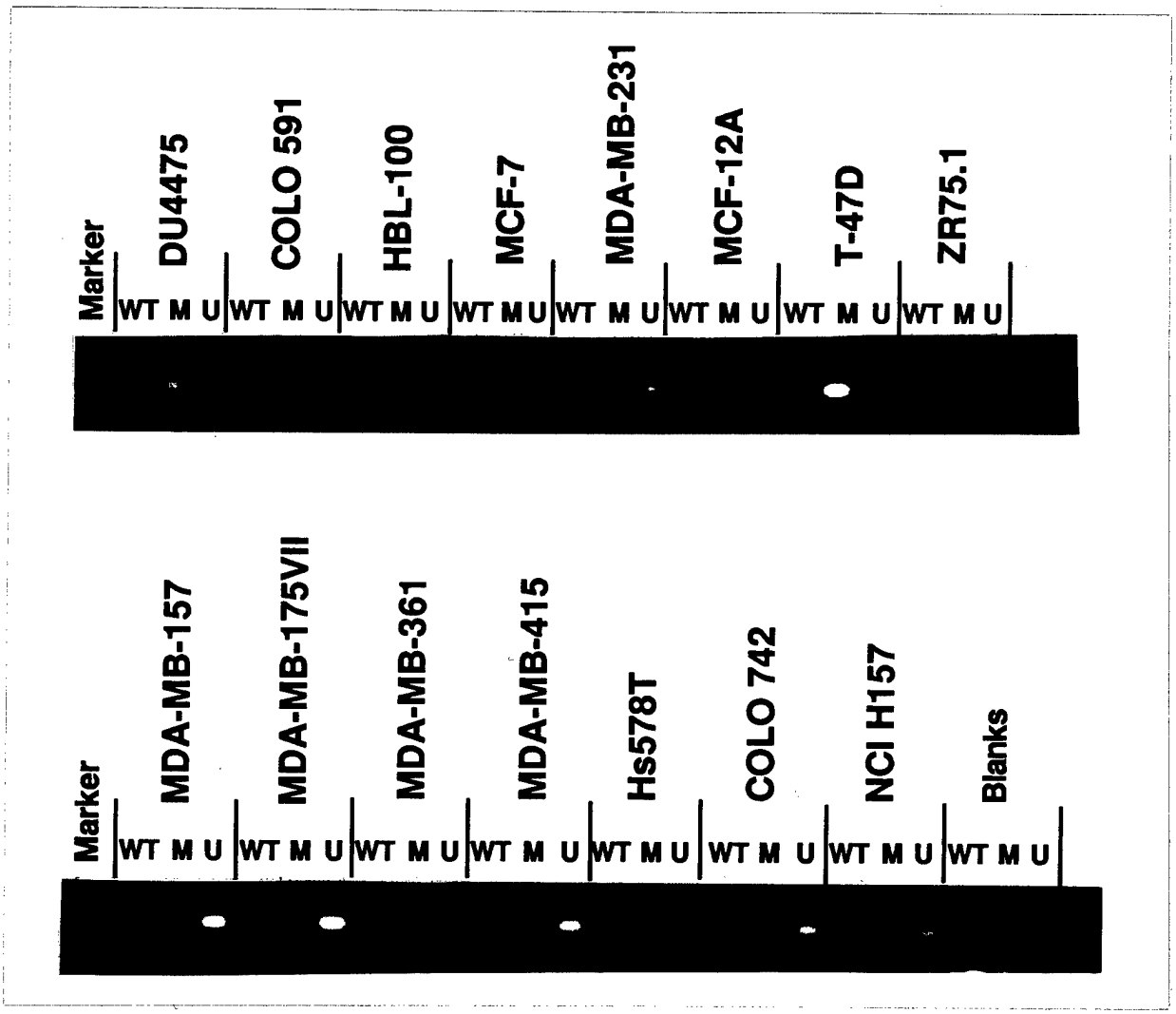

Figure 3 Methylation-specific PCR (MSP) of breast cancer cell lines at the p16 locus.

Following modification of cell line DNA with sodium bisulfite (converting all unmethylated cytosine's to uracil), it was subjected to PCR using primers from the promoter of the p16 gene specific for methylated (M) or unmethylated (U) DNA. A pair of primers specific for unmodified (WT) DNA were also used. PCR products were electrophoresed on a 2% agarose gel and the amplicons visualized with uv following ethidium bromide staining. Two of the cell lines, DU4475 and T-47D, showed amplification with the primers specific for methylated DNA and one of the cell lines, ZR75.1, showed amplification with the primers specific for both methylated and unmethylated DNA. The normal breast epithelial cell line, MCF-12A was shown to undergo weak amplification with the primers specific for unmethylated DNA on three different occasions. However, the ethidium bromide-stained amplicon is not clearly visible in this figure. Modified DNA derived from a breast cancer cell line, COLO 742 (not described in the analysis of cell cycle protein expression) and a lung cancer cell line, NCI H157, were also included in the panel as controls for unmethylated p16. The three lanes labeled "Blanks" contain all of the components of each of the three PCR mixtures minus DNA to indicate the absence of contamination.

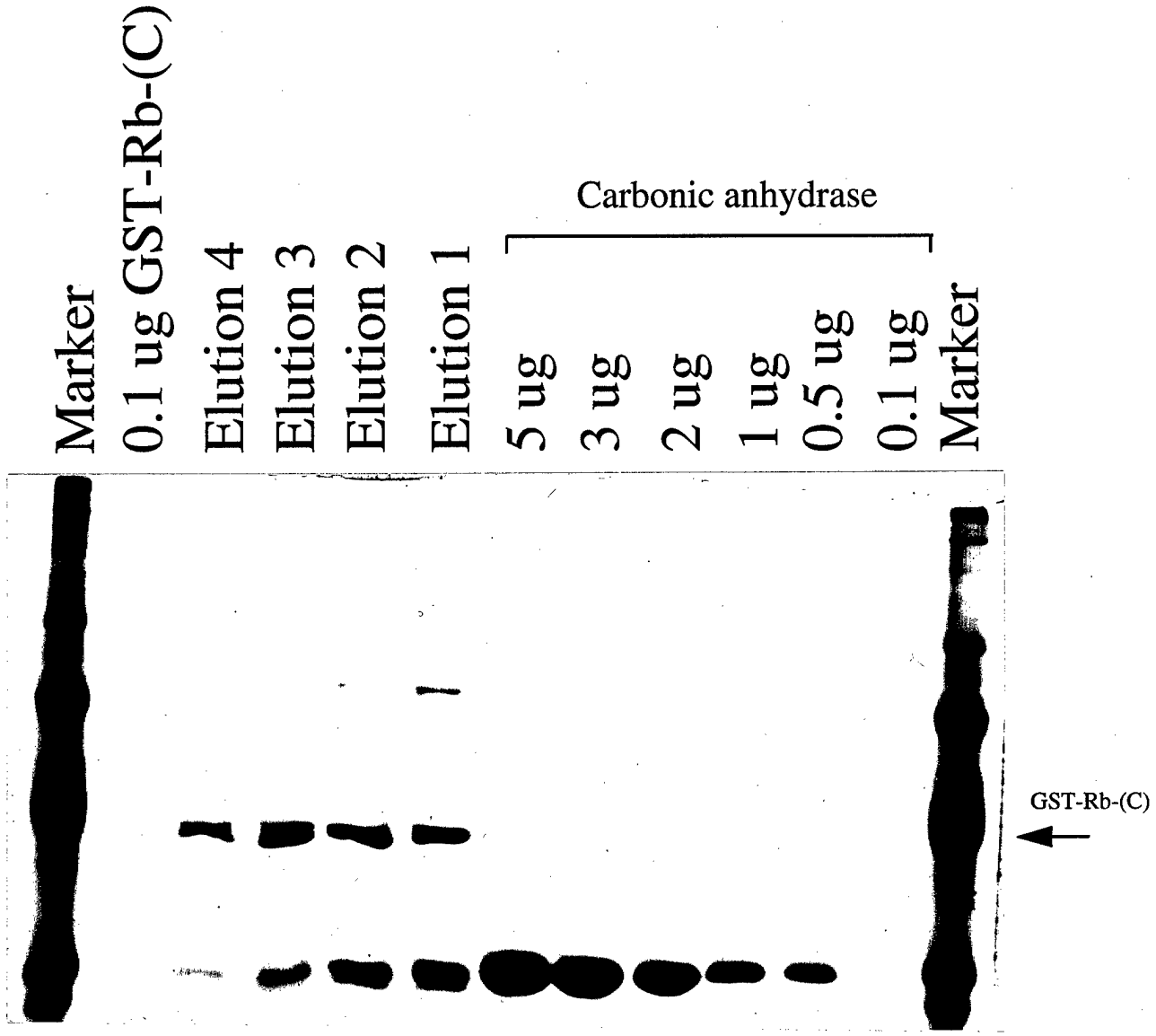

Figure 4 Purification of GST-Rb-(C) substrate.
Following binding of the GST-Rb-(C)-expressing E.coli lysates to glutathione-sepharose beads, the latter were washed and the GST-Rb-(C) fusion protein eluted in four sequential steps using reduced glutathione. Ten microliter aliquots of each of the four eluates were analyzed for purity and concentration by subjecting them to SDS-PAGE with 0.1 ug previously purified GST-Rb-(C) substrate (prepared by B. Grimison, Sclafani Lab) and known amounts of carbonic anhydrase protein standards.

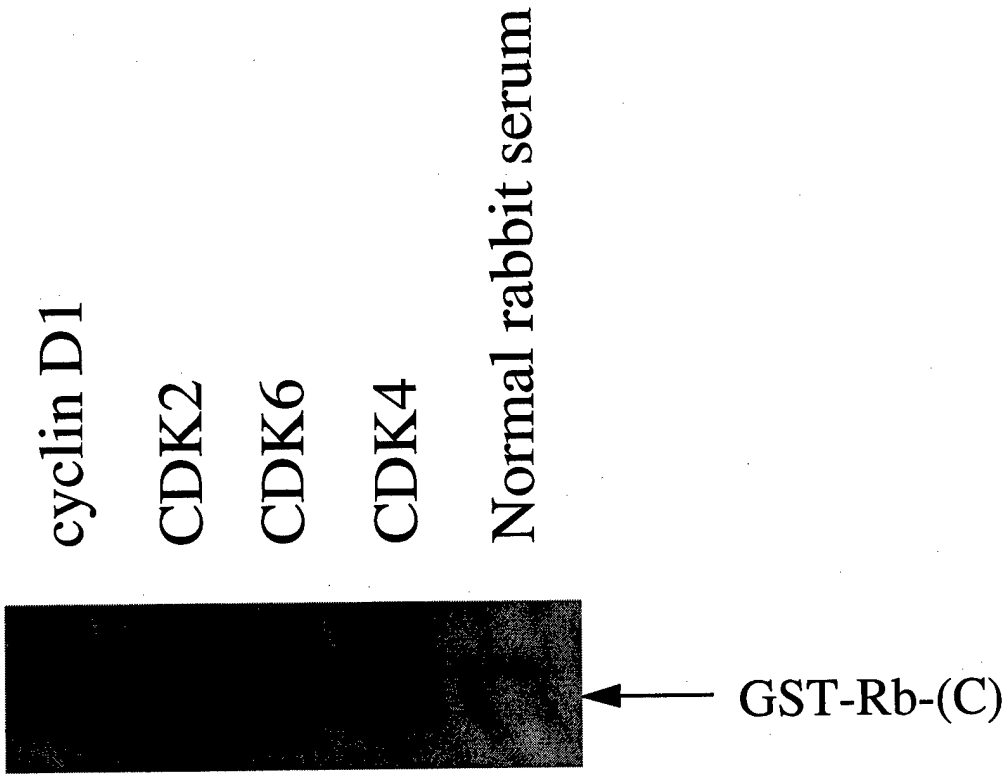

Figure 5 Immune complex kinase analysis of MCF-7 lysates. Following immunoprecipitation of MCF-7 lysates with cyclin D1, CDK2, CDK6, CDK4 and normal rabbit serum (as a negative control), the resulting complexes were washed and assayed for kinase activity using GST-Rb-(C) as substrate. The kinase reactions were stopped with SDS-PAGE loading buffer, boiled, subjected to SDS-polyacyclamide gel electrophoresis and exposed for autoradiography.

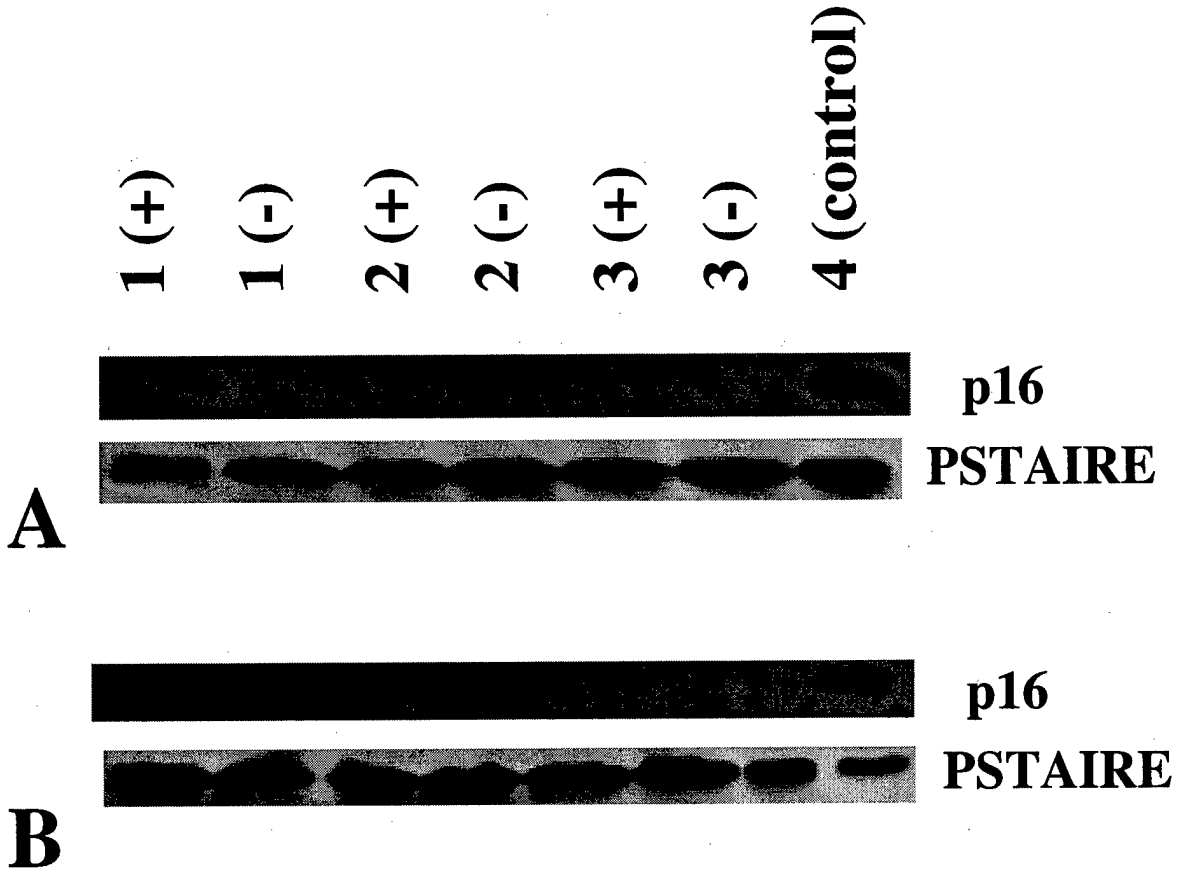

Figure 6 Transient transfection of MCF-7 (panel A) and ZR75.1 (panel B) with p16. Cells were transfected with DNA and grown in the presence (+) or absence (-) of doxycycline for 24 hours. 100 ug of each extract were then subjected to SDS-PAGE, transferred to nitrocellulose and probed with antibodies to p16 and PSTAIRE (as a loading control). Lanes 1(+) and 1(-), untransfected cells; lanes 2(+) and 2(-), transfected with pTET-SPLICE-p16 and pUDH15-1; lanes 3(+) and 3(-), transfected with pTET-SPLICE-p16 only; lane 4, HBL-100 (positive control for p16).